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Endothelin-1 receptor blockade as new possible therapeutic approach in multiple myeloma

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Summary

New effective treatments are needed to improve outcomes for multiple myeloma (MM) patients. Receptors with restricted expression on plasma cells (PCs) represent attractive new therapeutic targets. The endothelin-1 (EDN1) axis, consisting of EDN1 acting through EDN-receptor A (EDNRA) and B (EDNRB), was previously shown to be overexpressed in several tumours, including MM. However, there is incomplete understanding of how EDN1 axis regulates MM growth and response to therapy. Besides EDNRA, the majority of MM cell lines and primary malignant PCs express high levels of EDNRB and release EDN1. Similarly, bone-marrow microenvironment cells also secrete EDN1. Investigating the extent of epigenetic dysregulation of *EDNRB* gene in MM, we found that hypermethylation of *EDNRB* promoter and subsequent down-regulation of *EDNRB* gene was observed in PCs or B lymphocytes from healthy donors compared to EDNRB-expressing malignant PCs. Pharmacological blockade with the dual EDN1 receptor antagonist bosentan decreased cell viability and MAPK activation of U266 and RPMI-8226 cells. Interestingly, the combination of bosentan and the proteasome inhibitor bortezomib, currently approved for MM treatment, resulted in synergistic cytotoxic effects. Overall, our data has uncovered EDN1-mediated autocrine and paracrine mechanisms that regulate malignant PCs growth and drug response, and support EDN1 receptors as new therapeutic targets in MM.

Keywords: endothelin 1, endothelin receptors, multiple myeloma, bosentan, bortezomib.

Multiple Myeloma (MM) is a neoplastic plasma cell (PC) disorder that is characterized by clonal proliferation of malignant PCs in the bone marrow (BM), a monoclonal gammopathy, and a significant morbidity due to organ dysfunction (Palumbo & Anderson, 2011). MM is the second common haematological malignancy and, despite recent developments in novel therapies, such as immunomodulator drugs and proteasome inhibitors, it remains an incurable disease (Rajkumar, 2016). Although various genomic aberrations have been shown to provide PCs with the ability to proliferate in an uncontrolled manner, increasing evidence suggests critical roles for surface receptors with restricted expression in malignant PCs and for the BM microenvironment in mediating MM survival, proliferation and resistance to therapy (Bianchi & Munshi, 2015). Reciprocal growth factor exchanges between BM microenvironment cells and malignant PCs may, in turn, shape the BM microenvironment, composed of haematopoietic and non-haematopoietic cells, towards a protumourigenic phenotype (Anderson & Carrasco, 2011; Vacca *et al*, 2014; Ribatti *et al*, 2015). Therefore the mutual interactions between MM PCs and BM microenvironment cells – mediated by a growing array of cytokines, receptors and adhesion molecules – are currently the object of intensive investigation given their potential role as therapeutic targets (Podar *et al*, 2009; Shay *et al*, 2016).

The endothelin (EDN) family includes three, structurally similar, 21-amino acid peptides, named endothelin-1

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(EDN1), EDN2 and EDN3. EDN1 and EDN2 activate, with equal affinity, two G-protein coupled receptors, the EDN-receptors A (EDNRA) and B (EDNRB), while EDN3 binds EDNRB with lower affinity (Davenport *et al*, 2016). EDNs and their receptors, referred to as the "EDN axis", exert key physiological functions in normal tissues (Nelson *et al*, 2003; Maguire & Davenport, 2014). In particular, EDN1, originally isolated from endothelial cells (ECs), is considered the most potent vasoconstrictor in the human cardiovascular system (Maguire & Davenport, 2014). In the last decade, the EDN axis, especially EDN1 acting through EDNRA and EDNRB, has been implicated in the development of an increasing number of tumours, *via* an autocrine or paracrine activation of pathways involved in cell proliferation, migration, invasion, epithelial-mesenchymal transition, osteogenesis and angiogenesis. (Rosanò *et al*, 2013). As a consequence, the possibility of interfering with the EDN1 axis has been explored at pre-clinical and clinical levels in different cancer setting (Rosanò & Bagnato, 2016).

Although recent evidence suggests a role for the EDN1 axis in MM (Zhan *et al*, 2006; Vaiou *et al*, 2016), the underlying molecular mechanisms activated by EDN1 axis in MM remain to be elucidated. Therefore, our study aimed to: (i) analyse the expression of EDNRA and EDNRB and EDN1 release in malignant PCs, using both primary MM PCs and MM cell lines; (ii) assess whether malignant PCs and cells of the BM microenvironment express and release EDN1 as autocrine and/or paracrine growth factor; and (iii) test the possibility of targeting the EDN1 receptors in MM for therapeutic purposes.

Methods

Patients, samples and cell lines

BM samples were collected from patients ($n = 100$) with newly diagnosed MM and from volunteer healthy donors (HDs) ($n = 15$) during routine diagnostic assessments and at the time of BM harvest, respectively. Peripheral blood (PB) samples from MM patients ($n = 8$) and HDs ($n = 15$) were obtained in the same settings. Overall, BM and PB samples were collected from March 2010 to July 2016 at the Haematology and Bone-Marrow Transplant Unit of the Verona University Hospital and at the Haematology Unit of the Treviso Hospital. Both patients and HDs provided written informed consent for the collection of samples and subsequent analysis, as approved by our institutional Ethics Boards. Patient demographics, clinical characteristics and treatment are reported in Table I. The human MM cell lines U266, OPM-2, LP-1, KMS-12-PE, RPMI-8226 and the human breast cancer cell line MCF-7 (DSMZ, Braunschweig, Germany), were cultured in RPMI 1640 medium and in Eagle's minimal essential medium (MEM) respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) in 5% CO_2 at 37°C. Mycoplasma contamination was excluded by the Mycoplasma Species kit (EuroClone, Milan, Italy). Human BM fibroblasts and mesenchymal stromal cells (MSCs), provided by Dr. G. Bassi (Department of Medicine, Verona University), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% P/S. Adipocytes, osteocytes, chondrocytes and myocytes were obtained by differentiating MSCs as previously described (Mosna *et al*, 2010). Human umbilical vein endothelial cells (HUVEC), a gift from Prof. C. Lunardi (Department of Medicine, Verona University), were cultured in F-12K Medium (Gibco, ThermoFisher Scientific, Waltham, MA, USA), 0·1 mg/ml heparin, 0·03–0·05 mg/ml EC growth supplement (ECGS) (Sigma-Aldrich, Milan, Italy) and 20% FBS. Cell suspensions from tonsils were provided by MA Cassatella.

The mononuclear cell fraction was isolated from each BM or PB sample by Ficoll-Paque solution and freshly examined. In 10 cases $CD138^+$ PCs were purified from the mononuclear fraction of MM BM samples using a positive immuno-magnetic cell selection kit (magnetic-activated cell sorting [MACS], Miltenyi Biotec, Bologna, Italy) according to the manufacturers' instructions. The percentage of $CD138^+$ PCs obtained ranged from 95% to 99%. B lymphocytes were purified from buffy coat using the human B cell isolation kit II (MACS, Miltenyi Biotec, Bologna, Italy), according to the manufacturers' instructions.

Table I. Demographic and clinical characteristics of the 100 Multiple Myeloma patients enrolled in the study.

	N (%)
Median age, years (range)	66 (41–90)
Male/Female	55/45
Stage	
I	23 (23%)
II	22 (22%)
III	53 (53%)
Unclassified	2 (2%)
Acute renal failure	10 (10%)
Classification	
IgA	17 (17%)
IgG	62 (62%)
Light chain	16 (16%)
Non-secretory	5 (5%)
First line therapy	84 (84%)
VD/VTD/VMP	64 (76·2%)
MP/MPT	13 (15·4%)
VAD	3 (3·6%)
TD	3 (3·6%)
RT	1 (1·2%)
No therapy	13 (13%)
Lost to follow-up	3 (3%)

MP, melphalan, prednisolone; MPT, melphalan, prednisolone, thalidomide; RT, radiotherapy on bone lesion; TD, thalidomide, dexamethasone; VAD, vincristine, doxorubicin, dexamethasone; VD, bortezomib, dexamethasone; VMP, bortezomib, melphalan, prednisolone; VTD, bortezomib, thalidomide, dexamethasone.

RNA isolation and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated from MM cell lines, primary MM CD138$^+$ PCs, HUVEC, and BM stromal cells (fibroblasts, MSCs, adipocytes, osteocytes, chondrocytes and myocytes) using the SV Total RNA Isolation System (Promega, Milan, Italy). Reverse transcription (RT) of 1 μg RNA was performed using the SuperScript III Reverse Transcriptase (Life Technologies, Monza MB, Italy) according to the manufacturers' instructions. The obtained cDNA was amplified by real-time PCR (qPCR) using the Fast SYBR Green Master Mix (Life Technologies, Monza MB, Italy) and intron-spanning primers for *EDNRA*, *EDNRB*, *EDN1* and glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*). Gene expression was quantified by comparative cycle threshold (Ct) method, by normalizing Ct values to the housekeeping gene *GAPDH* and calculating relative expression values. Sequences of the RT-qPCR primers used are available on request.

Western blotting

Following cells lysis, protein cell extracts (40 μg) were separated on a 10% sodium dodecyl sulphoxide-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblots were performed using the following primary antibodies: anti-EDNRA, anti-EDNRB (Abcam, Cambridge, UK), anti-EDN1 (GeneTex Inc., Irvine, CA, USA), anti-pospho-MAPK3/1 (ERK1/2), anti-MAPK3/1, anti-BCL2L1 (BCL-XL), anti-ACTB (ACTIN), anti-GAPDH (Cell Signaling Technology, Danvers, USA), and anti-tubulin (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Blots were then developed using enhanced chemiluminescence (Amersham; GE Healthcare Life Sciences, Milan, Italy) and images were acquired by Image Quant Las 4000mini (GE Healthcare Life Sciences, Milan, Italy).

Flow cytometric analysis

Cells were incubated with anti-EDNRA (Novus Biologicals, Littleton, CO, USA) or anti-EDNRB antibody (Abcam, Cambridge, UK). BM samples from MM patients, HDs and the mononuclear cell fraction from tonsils were also stained with 5 μl of the following antibodies: anti-CD38 PerCP, anti-CD138 APC, anti-CD45 APC-Cy7, anti-CD56 PE-Cy7, anti-CD19 FITC (Biolegend, London, UK). In order to distinguish different steps of B lymphocytes maturation, BM cells from HDs were also stained with anti-CD45 APC-Cy7, anti-CD19 FITC, anti-CD10 APC and anti-CD20 APC-Cy7, while HDs and MM patients PBMCs were stained with anti-CD45 APC-Cy7, anti-CD19 FITC, anti-CD20 PE-Cy7 anti-CD27 APC. Quantitative fluorescence analysis was performed by the FacsCanto flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) using FlowJo software (Tree Star, Inc. Ashland, OR, USA) to collect and analyse data.

Immunohistochemistry

Immunohistochemistry analysis of EDNRB expression in BM biopsies from MM patients was performed as previously described (Wülfing *et al*, 2003), with minor modifications. Human trophoblast specimens were used as positive controls.

Methylated DNA Immunoprecipitation (MeDIP) analysis

MeDIP was performed as previously described (Mohn *et al*, 2009), with minor modifications. Briefly, genomic DNA obtained from primary CD138$^+$ PCs of 10 MM patients, MM cell lines and B lymphocytes was sheared by sonication with an ultrasonic homogenizer (Bandelin Sonopuls HD 2070, Bandelin, Berlin, Germany), to produce random fragments ranging in size from 300 to 1000 bp. Samples were subsequently incubated with 5-methylcytosine (5mC) monoclonal antibody (clone 33D3, Merck Millipore, Milan, Italy) and Dynabeads Protein G (10003D, ThermoFisher, Waltham, MA, USA). The immunoprecipitated (IP) DNA was then recovered using the Pure Link PCR Purification Kit (K310002, Thermo Fisher, Waltham, MA, USA) and qPCR was carried out on both IP DNA and input (IN) DNA by using primers specific for control genes (*H19* insulator control region [ICR], positive control, always methylated; and *HIST1H3B*, negative control, always un-methylated) and 2 sets of primers (i.e. A and B) for *EDNRB* promoter region. Sequences MeDIP primers are available on request. The relative enrichment of target sequences after MeDIP, was then expressed as percentage over IN DNA.

Enzyme-linked immunosorbent assay (ELISA)

EDN1 levels were measured in conditioned media of HUVEC, BM MSCs, BM fibroblasts and MM PCs after 48 h of culture by using the commercially available EDN1 Quantikine ELISA kit (R&D System, Minneapolis, MN, USA). Briefly, 200 μl of supernatants were incubated in duplicate for 2 h at room temperature in 96-well microplates coated with specific antibody. After washing, samples were treated with substrate solution for 2 h. The enzymatic reaction was stopped and read within 30 min at 450 nm and the concentrations reported in pg/ml. Assay sensitivity was 0·207 pg/ml.

Cell viability assay

U266 and RPMI-8226 cell lines were plated in 96-well plates (Corning LifeSciences, Big Flats, NY, USA) and treated for 48 h with 10 μmol/l BQ123 or BQ788 (Bachem, Bubendorf, Switzerland) alone or in combination. U266 and RPMI-8226 cell lines were also cultured for 48 h in the presence of

bosentan (SML1265, Sigma Aldrich, Milan, Italy) at the concentration of 50–100 μmol/l and/or bortezomib (Janssen-Cilag, Beerse, Belgium; discarded patient drug) 1–5 nmol/l. Cell viability rates were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma Aldrich, Milan, Italy) according to the manufacturer's instruction. The effects of interaction of the two drugs were analysed according to the median-effect method of Chou and Talalay (1984) using the CalcuSyn Software (Biosoft, Cambridge, UK). The mean combination index (CI) values were evaluated and combination data were plotted as CI *versus* fraction affected (Fa), defining the CI variability by the Sequential Deletion Analysis method.

Statistical analysis

Expression of plasma membrane EDN-receptors with respect to controls was analysed using one-way analysis of variance, while differences among groups were tested by Pearson's chi-square and Fisher exact tests. Correlation between EDNRB plasma membrane expression and DNA methylation in $CD138^+$ PCs was evaluated by the nonparametric Spearman correlation test. Analysis of drug-treated *vs* vehicle-treated cells was determined using the Student's *t* test. Differences were statistically significant for P values $\leq 0{\cdot}05$. Patients were staged according to Durie and Salmon (1975) and response criteria were established according to Rajkumar *et al* (2014). Time-to-treatment (TTT) was defined as the time from diagnosis to first treatment or last follow-up in case of untreated patients; overall survival (OS) was defined as the time from diagnosis to death or last follow-up. Survival curves were calculated according to Kaplan and Meier, and differences were tested using the log-rank test. Statistical analyses were performed by GraphPad PRISM® version 5-0c (La Jolla, CA, USA), and Stata SE v.14.2 by StataCorp (College Station, TX, USA).

Results

Expression of EDN1 *receptors by MM PCs and normal, long-lived and/or reactive PCs*

To establish the existence of an EDN1 axis in MM, we first evaluated the expression of EDNRA and EDNRB in a panel of MM cell lines, including U266, RPMI-8226, OPM-2, KMS-12-PE and LP-1. Although both *EDNRA* and *EDNRB* genes and proteins were detected by RT-qPCR and Western blot (Fig 1A, B, D, E), flow cytometry revealed that while EDNRA was expressed on the plasma membrane in all cell lines (Fig 1C), EDNRB could be detected only on the plasma membrane of U266, RPMI-8226 and LP-1 (Fig 1F). For primary MM PCs, 100 BM samples obtained at first diagnosis were evaluated by flow cytometry. Malignant PCs were distinguished from normal, long-lived, residual PCs in MM BM samples based on their expression of CD56 but not of CD19 (Kumar *et al*, 2005; Jeong *et al*, 2012) (Figure S1A, B). EDNRA was constitutively expressed by all primary MM PCs (Fig 1G), while EDNRB was detected in 54 out of the 100 cases under examination (Fig 1H and Figure S2). To ascertain whether EDNRA and EDNRB (in particular) are constitutively expressed by normal, long-lived PCs, or, alternatively, they are abnormally up-regulated during neoplastic transformation, we next evaluated PCs in 15 BM samples from HDs, as well as in 5 inflamed tonsils, by flow cytometry. While EDNRA was constitutively expressed by normal, long-lived and reactive PCs, EDNRB was always absent (Table II). Noteworthy, also immature and mature BM B lymphocytes or naïve or memory PB B lymphocytes from 15 HDs were found to express EDNRA, but not EDNRB (Table II). Similarly, PB B lymphocytes from 8 EDNRB-expressing MM patients displayed EDNRA, but not EDNRB (Table II). Collectively, our data indicate that normal or reactive PCs constitutively express EDNRA but not EDNRB. In contrast, EDNRB is aberrantly up-regulated during neoplastic transformation, as revealed by its expression in malignant PCs (Fig 1H). In our series, $EDNRB^+$ and $EDNRB^-$ patients did not differ with regard to age, sex, disease classification, stage, presence of bone lytic lesions or renal failure (Table SI). Moreover, the expression of EDNRB, in terms of median mean fluorescence intensity (MFI) did not differ among patients with different stages of disease [stage I: 2·75 (1·59–19·38); stage II: 3·45 (1·54–7·64); stage III: 3·51 (1·44–81·22); $P = 0{\cdot}81$].

Hypomethylated EDNRB gene promoter locus is associated with aberrant expression of EDNRB *in malignant PCs*

Previous studies have uncovered that the aberrantly increased expression of EDNRB is associated with low methylation levels of CpG islands located at the *EDNRB* gene promoter in melanoma and bladder cancer cells (Pao *et al*, 2001). Therefore, we investigated whether *EDNRB* expression in MM is regulated by epigenetic mechanisms. We measured the extent of methylation in a CpG island located in a highly CG-rich region of the *EDNRB* locus, spanning from position −792 to +451 with respect to the classically defined *EDNRB* transcription start site (TSS) (Knight *et al*, 2009; Welch *et al*, 2013). This CpG island was analysed by using 2 different primer sets (i.e. A and B) amplifying a region upstream (from +205 bp to +15 bp) and downstream (from −216 bp to −412 bp) of the TSS, respectively (Lo *et al*, 2002; Welch *et al*, 2013). Interestingly, we found that the two $EDNRB^-$ MM cell lines (i.e., OPM-2 and KMS-12-PE) were highly methylated at the *EDNRB* gene promoter regions, while MM cell lines displaying EDNRB on the surface (i.e. U266, RPMI-8226 and LP-1) showed lower DNA methylation levels (Figs 1F and 2A). To ascertain whether the same correspondence between EDNRB surface expression and gene promoter

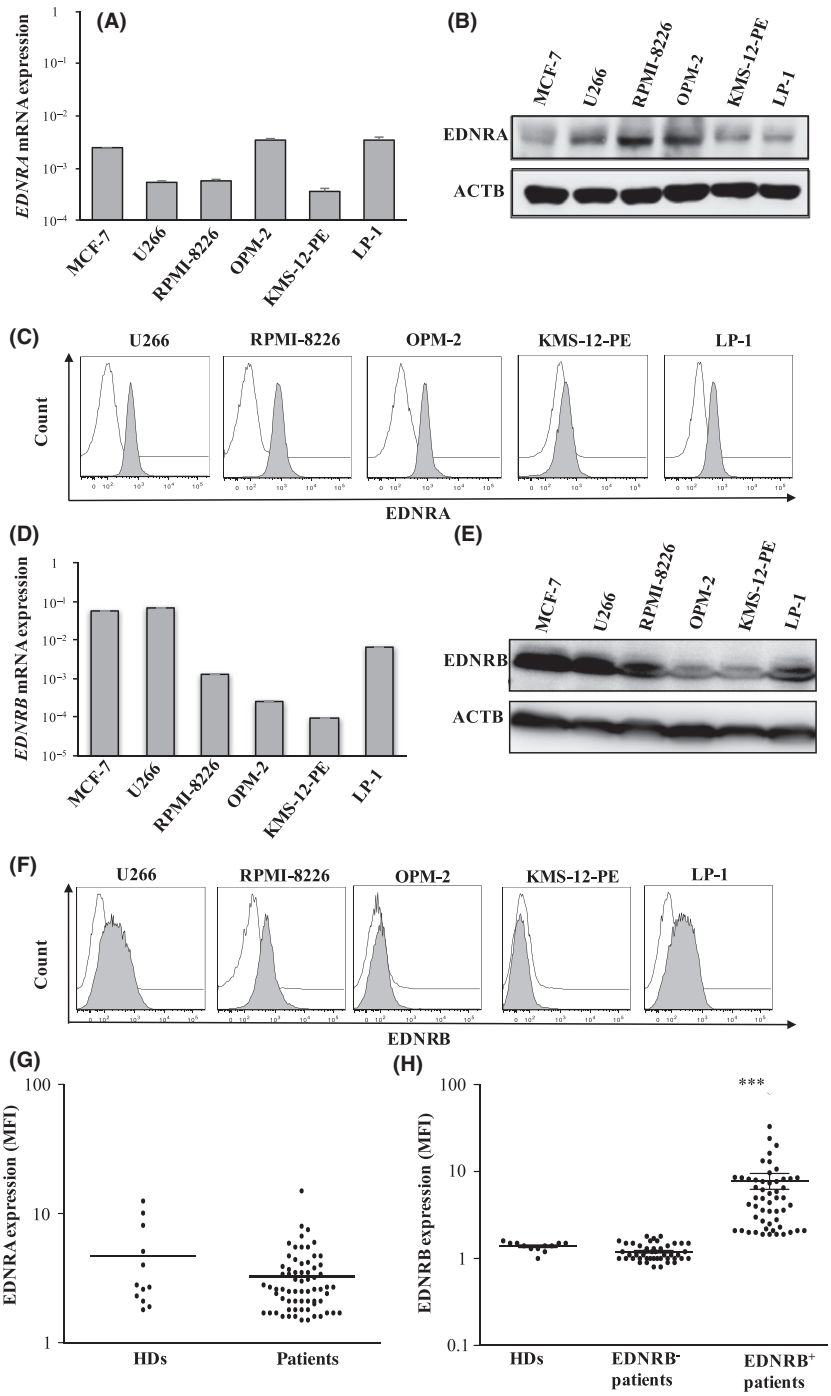

Fig 1. Expression of EDN-receptors by MM cell lines and primary MM plasma cells. mRNA and protein expression of *EDN*-receptors in MM cell lines. (A and D): RT-qPCR of *EDNRA* and *EDNRB* mRNA, respectively. Data represent the mean value ± standard deviation of 3 independent experiments. (B and E): EDNRA and EDNRB protein expression by Western blot analysis. MCF-7: breast cancer cell line used as positive control. (C and F): flow cytometry analysis of plasma membrane EDNRA and EDNRB. Open histogram: control (secondary antibody only); filled grey histogram: specific staining. (G and H): scatter plot distribution of EDNRA and EDNRB expression, respectively, by plasma cells from healthy donors (HDs) and MM patients. The cut-off positivity for EDNRA and EDNRB (i.e., ≥1·5) was established based on the ratio between the mean fluorescence intensity (MFI) of each sample and its control. ***$P < 0{\cdot}001$.

methylation levels also occurs in primary MM PCs, we analysed $CD138^+$ PCs isolated from BM samples obtained from $EDNRB^+$ ($n = 4$) and $EDNRB^-$ ($n = 6$) MM patients. B lymphocytes obtained from PB samples of HDs ($n = 3$) were also evaluated. As shown in Fig 2B and C, $CD138^+$ PCs with the lowest methylation levels at the CpG island of the *EDNRB* gene promoter displayed the highest expression of EDNRB. Conversely, $CD138^+$ PCs with elevated DNA methylation levels displayed no, or very low expression of EDNRB. The inverse correlation between EDNRB expression and DNA methylation in $CD138^+$ PCs is statistically significant and is more stringent for the downstream region (Spearman $r = -0{\cdot}8511$, $P = 0{\cdot}0018$) than for the upstream one of the CpG island (Spearman $r = -0{\cdot}7720$, $P = 0{\cdot}0089$) (Figure S3). Overall, these data appear in agreement with an inhibitory role of DNA methylation in the control of EDNRB expression. Data also indicate that, in a proportion of MM cases, malignant PCs express EDNRB as a consequence of an

Table II. Flow cytometry analysis of plasma membrane expression of EDNRA and EDNRB in BM and tonsil plasma cells, BM and PB B lymphocytes from HDs and from PB B lymphocytes of EDNRB$^+$ MM patients.

	EDNRA	EDNRB
HDs plasma cells		
Bone marrow (n = 15)	+	–
Tonsil (n = 5)	+	–
HDs B lymphocytes (n = 15)		
BM immature	+	–
BM mature	+	–
PB naïve	+	–
PB memory	+	–
MM PB B lymphocytes (n = 8)	+	–

BM B lymphocytes were defined as immature (CD19$^+$CD10$^+$) or mature (CD19$^+$CD20$^+$); PB B lymphocytes were defined as naïve (CD19$^+$CD20$^+$CD27$^-$) or memory (CD19$^+$CD20$^+$CD27$^+$).

epigenetic dysregulation occurring at the corresponding promoter region.

BM microenvironment cells and malignant PCs express and release EDN1

The BM microenvironment is normally composed of vascular ECs and stromal cells (Ribatti *et al*, 2015). To establish whether the BM microenvironment may be a source of EDN1, we initially analysed the expression of *EDN1* mRNA, either in HUVEC (used as a surrogate of BM vascular ECs, i.e., positive control), or in BM stromal cells, by RT-qPCR. All BM stromal cells were found to express *EDN1* mRNA, albeit at lower levels than HUVEC (Fig 3A). Moreover, since the EDN1 axis acts also in an autocrine fashion in different cancer cells (Rosanò *et al*, 2013), we evaluated whether malignant PCs from MM patients or MM cell lines express *EDN1* mRNA. As shown in Fig 3B, primary MM PCs and MM cell lines were found to express *EDN1* mRNA at levels comparable to BM stromal cells. Significantly, RT-qPCR data were also confirmed at the protein level, as shown by Western blot and ELISA (Fig 3C, D), demonstrating that both BM microenvironment cells (i.e., HUVEC, MSCs) and malignant PCs (primary and cell lines) produce and release EDN1. Collectively, these data indicate that both BM microenvironment cells and malignant PCs may represent EDN1 sources, and that, in MM, the EDN1 axis may act in a paracrine and/or autocrine fashion.

EDN1 *affects viability of MM cells in an autocrine fashion*

Next, we evaluated whether EDN1 could act as an autocrine growth factor in malignant PCs, using U266 and RPMI-8226 cell lines, which express both EDNRB and EDNRA. Given that these cells produce considerable levels of EDN1 (Fig 3B, C), we hypothesized an autocrine role for the EDN1 axis in the same cells. To test this hypothesis, we cultured cells in the presence of specific EDNRA and EDNRB antagonists, namely BQ123 and BQ788, respectively, alone and/or in combination. Consistent with our assumption, both BQ123 and BQ788, used alone and/or in combination, significantly reduced the viability of U266 and RPMI-8226 cell lines (Fig 4A), demonstrating an autocrine role for the EDN1 axis in the MM setting. To further demonstrate the autocrine role of EDN1 we tested bosentan, an orally active dual EDN-receptor antagonist, which is currently licensed in Europe and USA for the treatment of pulmonary arterial hypertension (Davenport *et al*, 2016), in the same MM cell lines. Bosentan reduced U266 and RPMI-8226 cell viability over a 48-h culture (Fig 4A and B), being significantly more effective than BQ123 and BQ788 used in combination (Fig 4A). Notably, bosentan displayed a dose-dependent effect in both cell lines, and exerted a stronger anti-proliferative effect in RPMI-8226 (50 μmol/l 50% inhibitory concentration) than in U266 (50 μmol/l 75% inhibitory concentration) cells (Fig 4B).

The dual EDN1 *receptor antagonist, bosentan, synergizes with bortezomib to inhibit MM cell viability*

Given that the EDN1 axis may act as an escape mechanism to treatment with the proteasome inhibitor bortezomib (Vaiou *et al*, 2016), which is currently approved as upfront treatment in MM (Moreau *et al*, 2012), we next sought to verify whether bosentan could potentiate the effect of bortezomib. Although both cell lines were sensitive to 5 nM bortezomib, the combined exposition to 100 μmol/l bosentan and 5 nmol/l bortezomib, further impaired the viability of U266 and, at higher levels, RPMI-8226 cell lines, exerting stronger effects than bosentan or bortezomib used alone at the same concentration (Fig 5A). Moreover, bosentan and bortezomib were found to have synergistic effects on cell viability based on CalcuSyn Software (Fig 5B and Table SII). Taken together, our results show that the EDN1 axis might represent a new therapeutic target in MM and that bosentan in combination with bortezomib-based therapy could be effective in enhancing cytotoxic effects in MM.

Bosentan exerts anti-proliferative effects in MM cells by inhibiting the MAPK/MAPK3/MAPK1 *and pro-survival pathways*

To evaluate the mechanisms underlying the anti-proliferative effects exerted by BQ123, BQ788 and bosentan, we examined the MAPK/MAPK3/MAPK1 signalling pathway involved in EDN1-mediated protumourigenic functions (Rosanò *et al*, 2013). We showed variations of phosphorylated MAPK3/MAPK1 in U266 and RPMI-8226 cells treated with 10 μmol/l BQ123 and/or BQ788, or 100 μmol/l bosentan for 48 h. We also evaluated the combinatory effects of bosentan and 5 nmol/l bortezomib under the same experimental conditions. As shown by Western blotting, elevated basal levels of

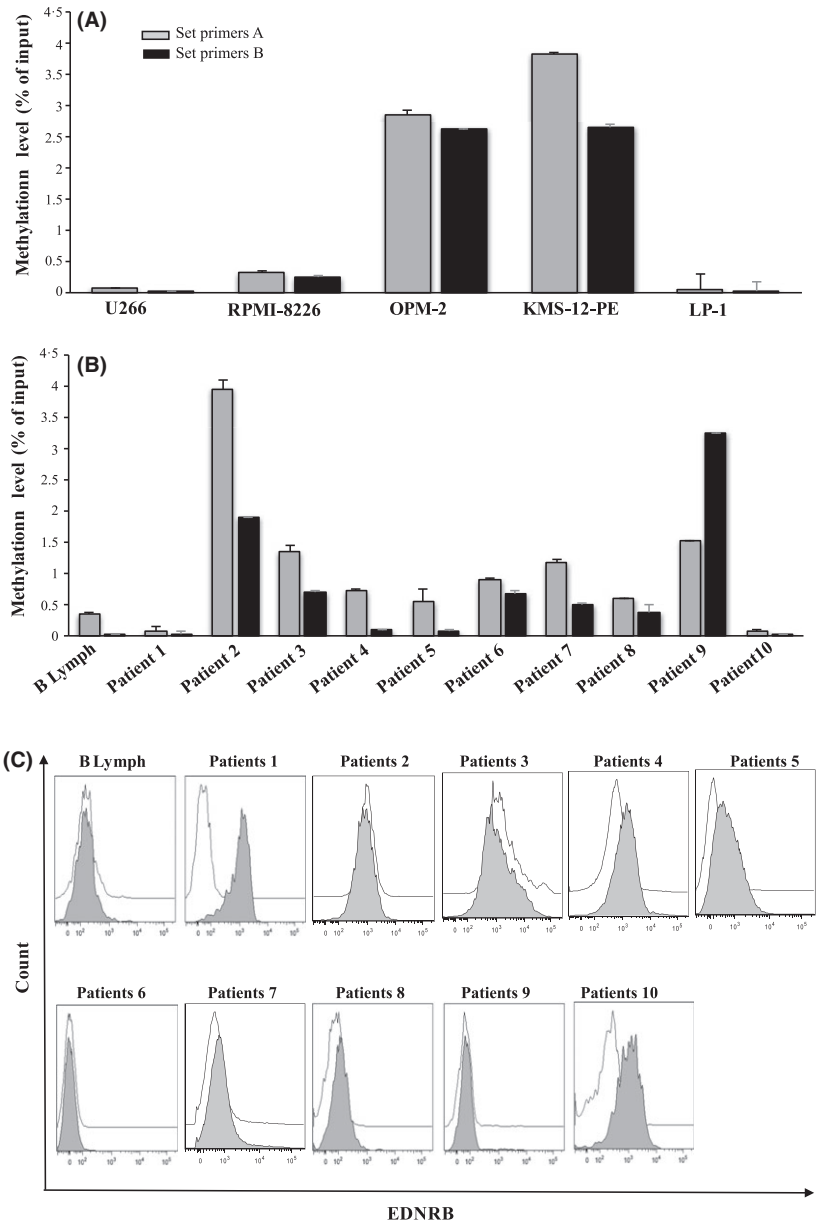

Fig 2. DNA methylation levels of the *EDNRB* gene and surface EDNRB expression in MM cell lines and primary MM plasma cells. Methylation levels expressed as a percentage of methylation of the *EDNRB* promoter (A and B) and flow cytometry analysis of the corresponding surface EDNRB expression (C), in MM cell lines (A) and primary MM plasma cells (B). Data in (A) represent the mean value ± standard deviation of 3 independent experiments, each performed in triplicate compared to the control. In (B) error bars represent standard errors calculated from triplicate qPCR reactions.

total MAPK3/MAPK1 were found in both cell lines under resting conditions (Fig 6A). Bosentan reduced phosphorylated MAPK3/MAPK1 levels in a substantial fashion in both cell lines, while BQ123 and BQ788 used in combination were more effective in RPMI-8226 but not in U266 cells (Fig 6A). In spite of a down-regulation effect exerted by bortezomib on total MAPK3/MAPK1 expression, bosentan was also found to potentiate the bortezomib-induced reduction of phosphorylated MAPK3/MAPK1 levels in U266 cells (Fig 6A). Finally, bosentan alone down-modulated BCL2L1 expression in RPMI-8226 cells, inducing a full reduction of BCL2L1 in both cell lines when used in association with bortezomib (Fig 6B). Taken together, our results confirm the potential role of bosentan alone and/or in combination with bortezomib, in the treatment of MM, further indicating its capacity to interfere with major signalling and anti-apoptotic pathways.

Discussion

Although a protumourigenic role of EDN1 was previously described in a number of solid tumours (Bagnato *et al*, 2011), as well as in B-cell chronic lymphocytic leukaemia (Maffei *et al*, 2014), little is known regarding the eventual existence of the EDN-axis in MM (Vaiou *et al*, 2016).

In this study, we report that primary MM PCs express EDN1 receptors, and that MM PCs themselves and BM microenvironment cells produce EDN1. Interestingly, we found that EDN1 is released in significant amounts not only by ECs, which are usually overrepresented in MM, but also

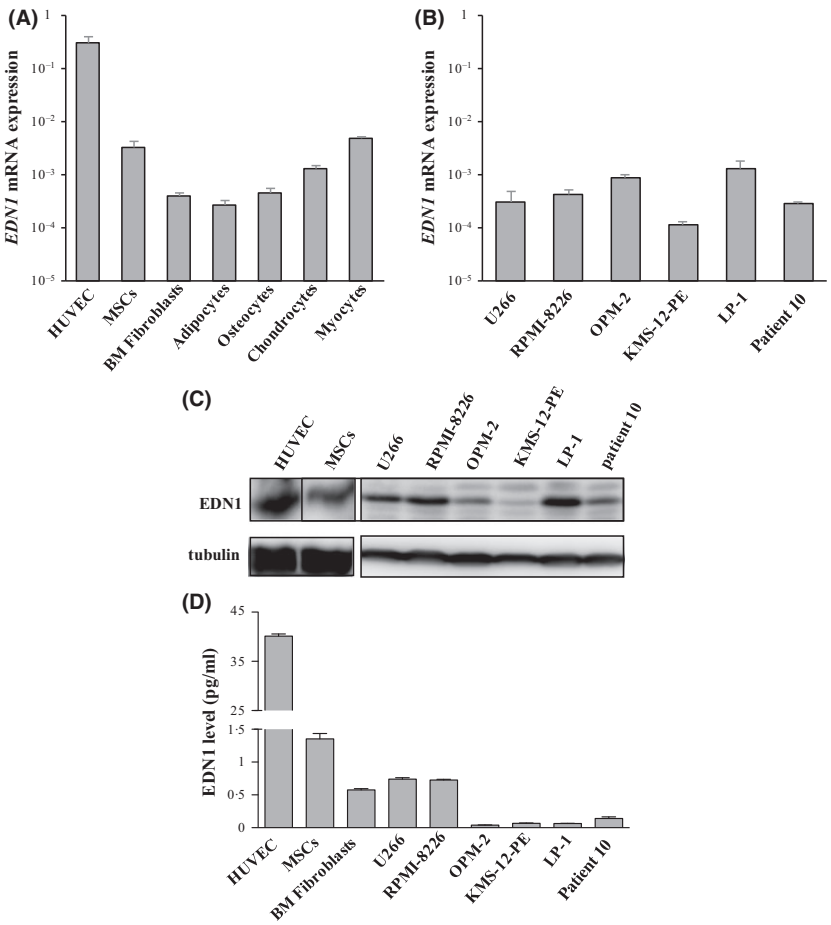

Fig 3. BM stromal cells, MM cell lines and primary MM PCs express and produce EDN1. RT-qPCR analysis of *EDN*1 mRNA expression normalized on *GAPDH* mRNA in: (A) bone marrow (BM) stromal cells and (B) MM cell lines and primary MM plasma cells (representative case). Data represent the mean value ± standard deviation (SD) of 3 independent experiments. (C) Western blot analysis of EDN1 in BM stromal cells, MM cell lines and primary MM PCs (representative case). (D) EDN1 levels measured by ELISA in 48 h-supernatants from human umbilical vein endothelial cells (HUVEC), BM stromal cells, MM cell lines and primary MM PCs (representative case). Data represent mean value ± SD of two independent experiments each performed in duplicate.

by other cellular components of the BM microenvironment, including MSCs (Vacca *et al*, 2014).

With regard to EDN1 receptors, surface EDNRB was detectable only in a proportion of cases, being evident in 54% of MM BM samples and in 60% of MM cell lines examined, while EDNRA was constitutively expressed by all malignant PCs. Based on these data, and on the lack of surface EDNRB expression in normal long-lived BM or tonsil PCs and immature and mature B lymphocytes, we interpreted EDNRB expression to be aberrant, at least in a number of MM cases. Interestingly, previous studies had demonstrated that epigenetic dysregulation is an important contributor to MM pathogenesis, with global DNA methylation changing significantly during disease progression (Walker *et al*, 2011), as a consequence of lesions in gene-encoding histone methyltransferases and DNA methylation modifiers (Pawlyn *et al*, 2016). Given that up-regulation of EDNRB surface expression is due to an altered methylation status of the promoter gene (Pao *et al*, 2001), we hypothesized that the EDNRB expression could also be regulated by epigenetic mechanisms in MM. Consistent with this hypothesis, we demonstrated that the *EDNRB* promoter locus of $EDNRB^+$ MM samples or cell lines displayed a lower methylation rate than $EDNRB^-$ primary MM samples, MM cell lines and B lymphocytes, suggesting a role for EDNRB as a MM biomarker.

Data concerning the function of EDNRB seem relatively controversial. Indeed, EDNRB has been reported to behave as either a tumour suppressor (Zhao *et al*, 2009; Schussel *et al*, 2013) or a protumourigenic (Lahav, 2005; Cruz-Muñoz *et al*, 2012) gene, depending on its actual function in the corresponding normal tissues. Accordingly, EDNRB was found to be down-regulated or absent in tumours of epithelial origin, whose normal counterparts express EDNRB with anti-proliferative and regulatory functions (Tao *et al*, 2012), whereas it appears overexpressed in malignancies (e.g. melanoma) whose normal counterparts express EDNRB as a mediator of pro-survival signals (Saldana-Caboverde & Kos, 2010).

In fact, MM cell lines showed a decreased viability when exposed to the EDNRB antagonist BQ788, concordantly with data previously obtained in melanoma (Lahav *et al*, 1999; Saldana-Caboverde & Kos, 2010), glioma (Paolillo *et al*, 2010) and glioblastoma (Egidy *et al*, 2000). A recent study reports the role of EDNRB in two MM cell lines expressing EDN1 (Vaiou *et al*, 2016). Interestingly, those authors also found that EDNRB mediates pro-survival signals, as the exposition of MM cell lines to the EDNRB antagonist BQ788

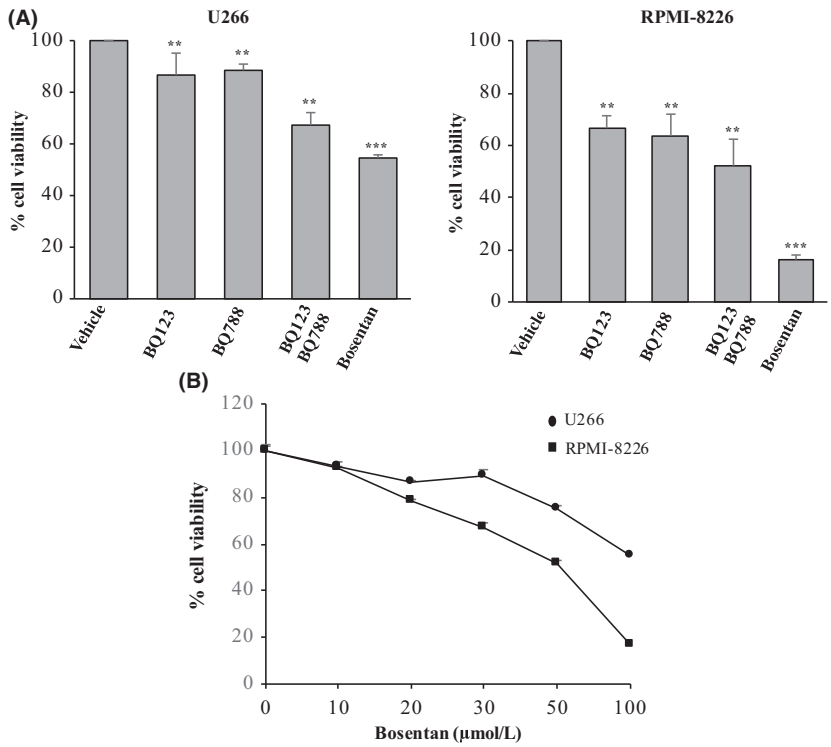

Fig 4. BQ123, BQ788 and bosentan affect viability of MM cell lines. (A) U266 and RPMI-8226 cells were incubated in the presence of 10 μmol/l BQ123 and/or BQ788, and 100 μmol/l bosentan for 48 h. Cell viability was determined by the MTT assay. Data represent mean values ± standard deviation (SD) of 5 independent experiments, each one performed in triplicate. $^{**}P < 0{\cdot}005$ and $^{***}P < 0{\cdot}001$ *versus* control. (B) U266 and RPMI-8226 cell viability after a 48 h treatment with increasing concentrations of bosentan. Each point represents the mean ± SD of 5 independent experiments, each one performed in triplicate.

decreased cell viability and reduced the EDN1-mediated resistance to bortezomib (Vaiou *et al*, 2016). Based on these findings, and on our current data regarding the expression of EDNRB by PCs from a proportion of patient BM samples, it would be conceivable to anticipate a negative prognostic significance for EDNRB in MM. Unfortunately we couldn't observe a significant difference in terms of TTT or OS between $EDNRB^+$ and $EDNRB^-$ patients (not shown). However the cohort that we analysed was therapeutically treated in a quite heterogeneously manner (Table I), thus making it difficult to establish whether $EDNRB^+$ patients had a worse outcome (eventually due to an increased resistance of their disease to bortezomib) than those expressing EDNRA only. Therefore, the prognostic role of EDNRB will be clarified only by prospective studies analysing larger cohorts of homogeneously treated patients. Interestingly, while in melanoma the extent of EDNRB expression was shown to parallel with disease aggressiveness (Demunter *et al*, 2001), we observed an elevated EDNRB expression even in the initial stages of MM, therefore allowing us to speculate that a very early epigenetic event may occur in malignant PCs.

The expression of either EDNRA only, or EDNRA and EDNRB, by MM PCs might have immediate therapeutic implications, given that EDN1 receptor antagonists are currently used in clinics, although not in non-haematological settings (Maguire, 2016). In fact, we report that the dual EDN- receptor antagonist, bosentan, was effective in reducing viability of MM cell lines *in vitro*, even showing synergistic effects with bortezomib. As a consequence, besides being highly effective, the combination of the two drugs might reduce the toxic effects of bortezomib that usually occur *in vivo* by allowing the reduction of bortezomib dosage (Argyriou *et al*, 2008). Interestingly, similarly to other drugs administered in MM, bosentan is also expected to act on the BM microenvironment, mostly by targeting EDNRB-expressing ECs (Spinella *et al*, 2014) and reducing angiogenesis.

The MAPK/MAPK3/MAPK1 signal transduction pathway has been demonstrated to significantly contribute to MM cell growth and survival, as well as to angiogenesis and to the development of drug resistance within the BM microenvironment *in vitro* and *in vivo* (Kim *et al*, 2010). Interestingly, EDN1 was shown to mediate protumourigenic functions through the same pathway (Rosanò *et al*, 2013), therefore anticipating a possible MAPK3/MAPK1-mediated EDN1 pro-survival role even in MM.

Our data have indirectly indicated that in U266 and RPMI-8226 cell lines the MAPK/MAPK3/MAPK1 pathway is strongly involved in mediating the autocrine pro-survival signals exerted by EDN1 through EDNRA and EDNRB. Accordingly, BQ123 and BQ788 in combination and, mostly, bosentan, were found to effectively down modulate *in vitro* phosphorylated MAPK3/MAPK1, as already observed in a number of solid tumours (Rosanò *et al*, 2013) and healthy tissues (Chen *et al*, 2009). In spite of these findings, we are aware that the therapeutic significance of our data has to be confirmed by an interventional study testing, in a mouse model of MM, the effects of bosentan alone and or in combination with bortezomib.

Finally, besides the autocrine function of EDN1 in MM, observations made in ECs and BM stromal cells also suggest

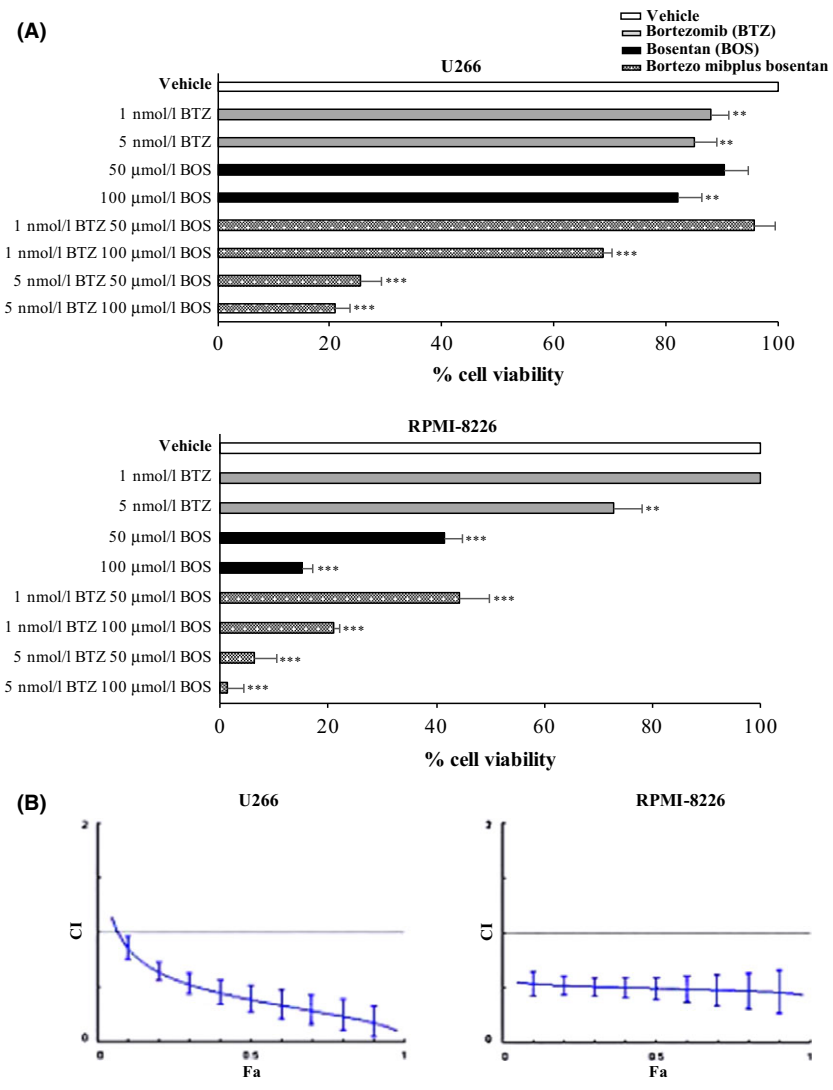

Fig 5. Bosentan and bortezomib synergistically decrease viability of MM cell lines. (A) U266 and RPMI-8226 cells were treated for 48 h with increasing concentrations of bosentan (BOS) and bortezomib (BTZ), used alone or in combination. Cell viability was determined by the MTT assay. Each point represents the mean ± standard deviation (SD) of 5 independent experiments, each one performed in triplicate. $^{**}P < 0{\cdot}005$ and $^{***}P < 0{\cdot}001$ *versus* control. (B) Fraction affected versus combination index (Fa-CI) plots displaying the interaction between bosentan and bortezomib in U266 and RPMI-8226 cells. Each point represents the mean ± SD of 5 independent experiments, each one performed in triplicate.

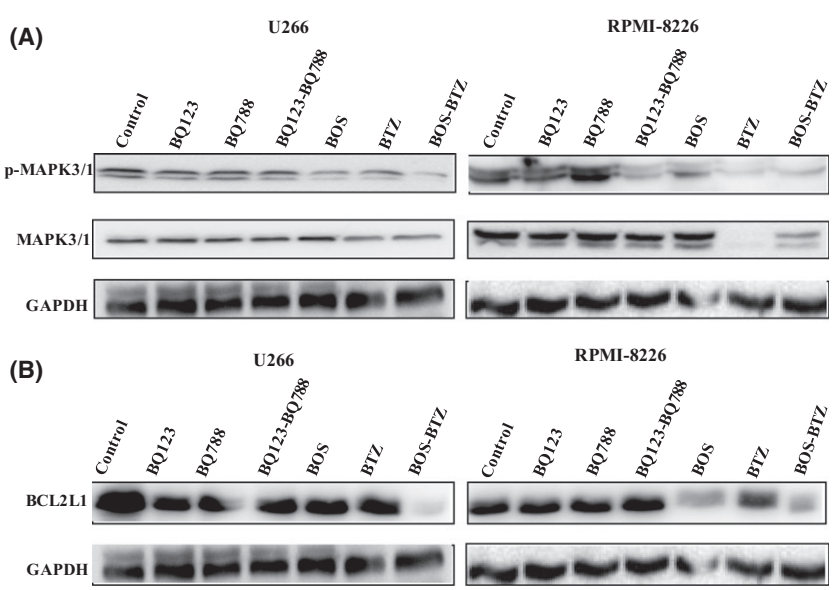

Fig 6. Bosentan and bortezomib modulate MAPK/MAPK3/1 phosphorylation and BCL2L1 expression. U266 and RPMI-8226 cells were treated with 10 µmol/l BQ123 and/or BQ788, 100 µmol/l bosentan (BOS) and 5 nmol/l bortezomib (BTZ), alone or in combination. Equivalent amounts of proteins (40 µg) were loaded and then immunoblotted with the following antibodies: (A) anti-p-MAPK3/1, anti-total MAPK3/1 and anti-GAPDH; (B) anti-BCL2L1 and anti-GAPDH.

an *in vivo* paracrine role for the EDN1 axis, especially in the MM stroma-dependent phase (Hallek *et al*, 1998). Further information on the paracrine role of EDN1 will derive from *in vitro* experiments targeted at evaluating the EDN1-mediated relationship between BM microenvironment cells and primary MM PCs.

In summary, our study uncovered an autocrine and paracrine EDN1-mediated mechanism of MM PCs survival together with the potential role of the EDN1 axis as a therapeutic target. Further pre-clinical and clinical studies are awaited in order to definitively establish the effectiveness and safety profile of EDN-receptor antagonists in MM.

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Authors contributions

RA, SC and TN performed the research and designed the research study, CA performed the research (experiments), RA performed the research, Bagnato A and RL analysed the data and wrote the paper, Bonalumi A, GM and ZL contributed clinical samples and essential clinical data, GA and ZA performed the research (experiments), SMT contributed essential tools, TM and SU contributed clinical samples, MF analysed the data, CMA and VF analysed the data and provided essential tools. TC designed the research study and wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Five-color flow cytometry analysis of PCs from an HD and a MM patient: sequential gating strategy. Panel A: representative HD; Panel B: representative MM patient. (A) Polygonal region 1 (R1) includes live cells on the basis of morphological parameter (FSC/SSC); (B) R2 excludes doublets from analysis; (C and D) R3 includes all $CD38^+$ cells, which are $CD138^+$ (R4). (E) CD45 and CD56 in normal *versus* neoplastic PCs. CD45 is weak in normal PCs, but variably expressed (dim to bright: e1, e2, e3) by MM PCs. PCs from HDs are usually $CD56^-$, while MM PCs express CD56 in the majority of cases. (F) PCs from HDs are usually $CD19^+$, while malignant PCs are $CD19^-$.

Fig S2. EDNRB immunohistochemical staining pattern in MM BM biopsy specimens. BM specimens from (A) a representative positive patient, and (B) a representative negative patient (200×). (C) in a human trophoblast specimen used as positive control (400×).

Fig S3. Correlation between EDNRB surface expression and methylation levels at the *EDNRB* promoter. Nonparametric Spearman correlation between specific EDNRB staining with respect to control (ΔMFI), and meDIP levels at the *EDNRB* gene, as measured in 2 regions amplified upstream (left) and downstream (right) with respect to the TSS. The Spearman correlation coefficient (r) and P-value are indicated.

Table SI. Demographic and clinical characteristics of $EDNRB^-$ ($n = 46$) *versus* $EDNRB^+$ ($n = 54$) patients.

Table SII. Combination indices (CI) in synergism experiments using bosentan and bortezomib in constant ratio. <0.1 = very strong synergism, 0.1–0.3 = strong synergism, 0.3–0.7 = synergism, 0.7–0.85 = moderate synergism, 0.85–0.9 = slight synergism.

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